Title

The invasive red swamp crayfish (*Procambarus clarkii*) increases infection of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*)

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Abstract

Emerging infectious diseases are increasingly recognized as a severe threat to wildlife. Chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (Bd), is considered one of the most important causes for the decline of amphibian populations worldwide. Identifying potential biological reservoirs and characterizing the role they can play in pathogen maintenance is not only important from a scientific point of view, but also relevant from an applied perspective (e.g. disease control strategies), especially when worldwide distributed invasive species are involved. We aimed (1) to analyse the prevalence and infection intensity of Bd in the invasive red swamp crayfish (*Procambarus clarkii*) across the western Andalusian region in Spain; and (2) to assess whether the presence of crayfish affects the prevalence and infection intensity of Bd in amphibians of Doñana Natural Space (DNS), a localized, highly protected area within the Andalusian region. First, we found that infection prevalence in crayfish guts was 1.5% regionally (four out of 267 crayfish were qPCR positive to Bd, all of them belonging to the same Andalusian population); qPCR positives were histologically confirmed by finding zoosporangia of Bd in gastrointestinal walls of the red swamp crayfish. Second, we found a higher prevalence of Bd infection in DNS (19% for crayfish and 28% for amphibians on average), a place with great diversity and abundance of amphibians. Our analyses showed that prevalence of Bd in amphibians was related to the presence of the red swamp crayfish, indicating that this crayfish could be a suitable predictor of Bd infection in co-occurring amphibians. These results suggest that the red swamp crayfish might be a possible reservoir for Bd, representing an additional indirect impact on amphibians, a role that had not been previously recognised in its invasive range.

Keywords

Amphibians, chytridiomycosis, Doñana, emerging infectious diseases, histological analysis, non-amphibian host, transmission.

Introduction

Globalized infectious diseases, particularly fungal pathogens, are one of the greatest threats to wildlife (Fisher et al. 2012). Amphibians are the most threatened vertebrates globally, being highly susceptible to climatic change, habitat fragmentation and overexploitation (Stuart et al. 2004). Moreover, they are affected by the most devastating panzootic so far, amphibian chytridiomycosis, which is closely related to amphibian declines (Voyles et al. 2009; GISD 2018;

Scheele et al. 2019). The amphibian-killing pathogen, *Batrachochytrium dendrobatidis* (hereafter "Bd"), infects keratinizing epithelial cells in amphibian skin (Berger et al. 1998; van Rooij et al. 2015), causing osmotic damage and often death (Daszak et al. 2003; Voyles et al. 2009). Bd is considered as one of the 100 worst invasive species worldwide (GISD 2018), whose origin has been recently attributed to Asia, and the pathways of its spread at the global scale are mainly linked to amphibian pet trade (O'Hanlon et al. 2018). Global trade is also resulting in the evolution of further hypervirulent fungal lineages across a diverse range of host species and biomes (Farrer et al. 2011).

Because pathogenicity of Bd is temperature-dependent, prevalence of Bd is usually higher in early spring and lower in late summer or autumn (Kriger and Hero 2007), which affects the temporal patterns of exposure (Walker et al. 2010; Xie et al. 2016). The highest rates of Bd-infection often coincide with the breeding season of many amphibian species during winter, making them more vulnerable (Gervasi et al. 2017). However, viability of the Bd pathogen decreases through the seasons in areas where water temperature rises over 30ºC and waterbodies dry up during the dry season (Piotrowski et al. 2004, Doddington et al. 2013). Thus, amphibians with a highly variable response to different levels of infection can be reservoirs of Bd, maintaining this pathogen in the environment (Woolhouse et al. 2001; Gervasi et al. 2017; Brannelly et al. 2018). Others have hypothesized on the saprophytic feeding of Bd, which is able to live without an amphibian host (Speare et al. 2001). In addition, in spite of being an amphibian fungal disease, there is increasing interest in evaluating the potential role of non-amphibian species, which co-occur in aquatic ecosystems, as reservoirs or carriers of chytridiomycosis (van Rooij et al. 2015). Identification of potential reservoirs and characterization of the role they play in Bd maintenance and disease dynamics is not only important from an evolutionary perspective, but can also provide important insights on disease control strategies.

The red swamp crayfish (*Procambarus clarkii*), native to north-eastern Mexico and south-central US, has been intentionally introduced worldwide, becoming the most widespread crayfish in the world (Oficialdegui et al. 2019). Over the last 45 years, southern European freshwaters have been widely invaded (Kouba et al. 2014), causing, among others, dramatic declines of many amphibian populations and negatively impacting their community structure (Ficetola et al. 2012), with an important decrease in amphibian richness (Cruz et al. 2006). The recent finding that the red swamp crayfish and other crayfish species can become infected by Bd in their native range (Louisiana, US), and potentially transmit infection to amphibians (McMahon et al. 2013, Brannelly et al. 2015a, but see Brannelly et al. 2015b), suggests the need for evaluating the role of invasive crayfish as carriers of the amphibian fungus in its invaded range. This is especially important in places where crayfish species are particularly prolific and colonize a wide range of aquatic environments where amphibians occur (GISD 2018).

The red swamp crayfish is especially abundant in the southern part of the Iberian Peninsula, including the Doñana Natural Space (DNS), a protected area located in western Andalusia where the conservation value of ponds for amphibians and other aquatic fauna and flora has been highlighted by many studies (Gómez-Rodríguez 2007; Díaz-Paniagua et al. 2010). Due to its commercial value, humans have intentionally translocated the red swamp crayfish among water bodies, promoting its rapid colonization across Europe (Oficialdegui et al. 2019). Thus, studies evaluating the role of the red swamp crayfish as potential reservoir in this area are of particular interest, as crayfish may rapidly disperse Bd over long distances and contribute to the global Bd pandemic in Europe. We conducted a study to explore these questions that comprised two sections. The first one was a regional survey across the western Andalusian region, with the goal of assessing the prevalence of infection and infection intensity of Bd in the red swamp crayfish in Andalusian streams and ponds (i.e., crayfish infection survey). The second one was a specific sampling in a localized, highly protected area within the Andalusia region (DNS), which is particularly rich in amphibian populations where Bd had been circulating through the environment (Hidalgo et al. 2012); our aim was determining whether Bd infection prevalence and intensity in amphibians was positively related to the presence of the red swamp crayfish in ponds (i.e., crayfish/amphibian interaction).

Methods

Study area

In the crayfish infection survey, we sampled 11 streams of the provinces of Seville, Cadiz and Huelva, in western Andalusia (South-western Spain) and three ponds within Doñana Natural Space (DNS) (Fig. 1A, see Table 1). The region is dominated by the Guadalquivir basin and characterized by a Mediterranean climate, with a long dry summer season, mean annual temperatures between <10°C and 18°C, and mean altitude of 200 m asl. Most streams in this Mediterranean region are intermittent, where streams have little flow during wet season water remains in puddles along the stream during dry season. Subsequently, for the study of crayfish/amphibian interaction, we sampled six different ponds in the Doñana Natural Space (DNS), a localized protected area within western Andalusia which is particularly important for amphibian breeding and conservation, in order to analyse the prevalence and infection intensity of Bd in relation to the presence of the red swamp crayfish (Fig. 1B). The DNS, situated in the mouth of the Guadalquivir River, comprises the Doñana Natural Park and the Doñana National Park, and is considered one of the largest and most important wetlands of Europe. It has been declared a UNESCO Biosphere Reserve, a Ramsar site, a Natural World Heritage Site, and part of the Natura 2000 network (see e.g. García-Novo and Marín-Cabrera 2005). As other very scarce Mediterranean temporary ponds, this system of more than 3,000 water bodies that integrate DNS is considered a priority habitat under the European Union Habitats Directive (Code 3170: European Commission DG Environment, 2007).

Sample collection

To address the first objective, adults of the red swamp crayfish were collected during late spring and early summer of 2015 from 14 sites distributed across the western Andalusian region in Spain (Fig. 1A, Table 1). For most sampling sites, 20 crayfish were captured using fyke nets (Table

1), subsequently euthanized by decapitation, and their gastrointestinal (GI) tracts were removed. Samples were fixed in 70% ethanol and maintained in the refrigerator until laboratory analysis. For the second objective, sampling was performed in six ponds of DNS between early March and early April of 2018 (Fig. 1B, Table 2 and 3). While crayfish were processed as for the first objective, amphibians were captured by hand nets and skin swabs were collected by gently rubbing a sterile cotton-tipped swab along their entire body. Subsequently, swabs were kept in Eppendorf vials with a drop of pure ethanol and maintained in the refrigerator until qPCR analysis.

Real-time PCR TaqMan assay for Bd-quantification

In the laboratory, to ensure no contamination between samples, we used disposable blades to dissect the GI tract from each crayfish. In order to avoid contamination by amphibian tissue inside the GI tissue of crayfish (i.e. in case crayfish had fed on infected amphibians), the sample was washed twice with deionized pressure water. By using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems), we extracted DNA from a small part of the GI tract of crayfish, and from swabs for amphibians. We quantified Bd DNA from GI tracts of crayfish and swabs using standard real-time Polymerase Chain Reaction (qPCR) procedures (Boyle et al. 2004). Amplifications were carried out in a 15µl volume reaction, which included a Bd-specific TaqMan probe (Chytr MGB) for the quantification of zoospore equivalents (ZE). We included amplification standards of 0.1, 1, 10 and 100 zoospore equivalents (ZE, where one ZE is equivalent to a single zoospore) prepared from an isolate of known cell density (IA042, Spain) and a negative control in each plate. All samples, diluted (1/10), standards and the negative control were analysed in duplicate. We considered Bd positive samples if both replicates resulted positive and the amplification curves had the expected sigmoidal shape (otherwise, a 1/100 dilution was made to prevent inhibition problems). In case of contradictory results, the sample was repeated a third time. Infection intensity was reported in zoospore equivalents (ZE) and log-transformed values to show results in graphs.

We considered ZE values of 0.1 (Cycle Threshold, CT < 37.0) or higher as positive for infection. Infection intensities are reported as the mean and standard error, unless otherwise noted.

Histological analyses

Only crayfish samples that were determined Bd positive by qPCR in the first objective (crayfish infection survey) were confirmed histologically. Samples were fixed in neutral buffered 10% formalin, embedded in paraffin and sectioned, before being stained with haematoxylin and eosin using routine methods (Drury & Wallington 1980).

Statistical analyses

While the prevalence of Bd^+ and infection intensity was only calculated in crayfish for the crayfish infection survey, we calculated the prevalence of Bd^+ and infection intensity in both crayfish and amphibians for the crayfish/amphibian interaction. For both objectives, we calculated prevalence as the proportion of Bd^+ with respect to the total number of sampled individuals (crayfish and amphibians), and infection intensity was calculated as Bd load [$\log_{10}$ (Bd load+1)] in each sample. In our models, we aimed to analyse whether the presence of crayfish could have an effect on the prevalence and infection intensity of Bd in amphibians. To do that, we pooled all amphibian species (see Table 3 for species sampled) and both sexes to increase sample size. As we captured sub-adults (non-mature phase) and adults, we used life stage as independent variable to control for different time of permanence in the water. Thus, we analysed the effects of presence/absence of crayfish, pond and amphibians' life stage (sub-adults and adults) on the prevalence of infection and infection intensity of amphibians. We used a Generalized Linear Model (GLM) with a binomial distribution to analyse the prevalence of infection and, in a second analysis only with Bd^+-amphibians, we used a linear model to analyse the infection intensity in amphibians from ponds in the localized protected area (DNS). The factor “pond” was nested within the factor “presence of crayfish in each pond”; both factors were included in the model because one level of the factor “pond” could not combine presence and absence of crayfish at the same time but ponds could differ in infection intensity. Normality of the residuals of both models was visually inspected and they did not differ from normality. All analyses were performed with JMP 12 software (SAS Inc.).

Results

Crayfish infection survey

Only four crayfish out of 267 were positive to Bd, which means a total prevalence of 1.5% (95% CI, 0.4 – 3.8). All Bd^{+}-individuals were found in one population, Olivargas stream (site 9, Fig. 1A), where the prevalence reached 20% (95% CI, 5.7 – 43.7) and average infection intensity was 4.1 ± 1.2 ZE (Table 1). After detecting qPCR Bd^{+}, histological analyses confirmed the presence of zoosporangia in all four infected GI walls of the red swamp crayfish (Fig. 2).

Crayfish/amphibian interaction

We sampled six different ponds (Fig. 1B) and captured a total of 37 crayfish and 165 amphibians of seven different species. A total of seven crayfish were qPCR-positive for Bd infection in their GI walls, which means a prevalence of 18.9% (95% CI, 8.0 – 35.2), and average infection intensity was 6.0 ± 2.0 ranging from 0.6 to 13.5 ZE. On the other hand, a total of 46 amphibians of three species (*Triturus pygmaeus*, *Pleurodeles waltl* and *Pelophylax perezi*) were qPCR positive for Bd infection (Table 3), reaching an average prevalence of 27.6% (95% CI, 21.2 – 35.4) and average infection intensity was 1.9 ± 0.6 ranging from 0.1 to 68.0 ZE (Table 2). Except for *P. perezi*, the other two species were widely represented in our samples. We rarely found positives in anurans (one individual of *P. perezi*) with prevalence of infection of 2.4% (95% CI, 0.1 – 12.6) on average. However, the prevalence of infection in urodelans was of 36.6 % (95% CI, 28.1 – 45.7), being the prevalence within species of 35.1% (95% CI, 24.5 – 46.8) for *T. pygmaeus* and 39.1% (95% CI, 28.1 – 45.7) for *P. waltl* (Table 3).

Our generalized linear model (binomial distribution) explained a 20.6% of total variance (generalized adjusted R^2) for the prevalence of infection. The prevalence of Bd in amphibians was affected by the presence of crayfish ($\chi^2 = 28.5$, $p<0.0001$), the pond ($\chi^2 = 8.8$, $p = 0.0121$) and life stage of amphibians ($\chi^2 = 4.8$, $p = 0.0280$) (Fig. 3A). However, the Bd infection intensity on amphibians was significantly affected by pond ($F = 2.90$, $df = 4.45$, $p = 0.0342$), but not by the presence of crayfish in the pond ($F = 1.83$, $df = 1.45$, $p = 0.1838$) or life stage ($F = 1.23$, $df = 1.45$, $p = 0.2737$) (Fig. 3B).

Discussion

Identification of non-amphibian hosts is crucial to understand the virulence, distribution, spread and persistence of Bd in aquatic ecosystems worldwide. McMahon et al. (2013) observed encystement of Bd within GI tracts of the red swamp crayfish from Louisiana, the native area of this species. Importantly, our study confirmed the histological evidence of Bd embedded within GI tracts of the red swamp crayfish in its invaded range, indicating that the red swamp crayfish could be a potential carrier of this disease wherever it invades. The presence of the red swamp crayfish could thus imply, besides a predatory effect on amphibians, an indirect effect through the transmission of Bd, promoting the decline of amphibians in Europe or elsewhere. Our results indicated that the prevalence of Bd-infection in amphibians was high when the red swamp crayfish co-occurred in the same pond and, to a lesser extent, showed effects of pond (site) and amphibian life stage, being the sub-adult stage more susceptible to be Bd positive. For infection intensity we only found a slight effect of pond but other non-tested variables (e.g. environmental variables or amphibian densities) could alter the infection intensity in amphibians. Therefore, our results suggest that non-amphibian species, and the presence of red swamp crayfish in particular, could play a crucial role in maintaining and spreading this emerging infectious disease (chytridiomycosis) in amphibians.

Due to its great impact on amphibians, chytridiomycosis is considered the worst infectious disease in vertebrates (Gascon et al. 2007; GISD 2018). Sudden high mortality rates in several amphibian species have been related to this emerging panzootic infectious disease in the last decades (Berger et al. 1998; Bosch et al. 2001; Daszak et al. 2003; Bosch and Martínez-Solano 2006; Skerrat et al. 2007; Scheele et al. 2019). In Andalusian streams, we found a prevalence of Bd of 1.5% in wild red swamp crayfish, which was similar to that found in a previous study in its native area (3.3% in the wild during spring) (Brannelly et al. 2015a). On the other hand, the red swamp crayfish in the localized highly protected area (DNS) showed a total prevalence of 18.9% (seven out of 37 sampled crayfish). Although our sampling size was too low to draw firm conclusions, it seems to exist a tendency to a higher prevalence of Bd in DNS compared to those from waterbodies across western Andalusia. It is well known that the infection of Bd is context-dependent on the amphibian host (Scheele et al. 2017), the fungal virulence (Fisher et al. 2009), and environmental determinants such as temperature, altitude, seasonality and/or rainfall (Berger et al. 2004; Kriger and Hero 2007; Walker et al. 2010; Doddington et al. 2013; Ruggeri et al. 2018). As presence of Bd is higher at low temperatures (Berger et al. 2004), spatial differences in Bd prevalence found between sampled areas (streams regionally and localized protected area) could be rather due to the sampling timing because crayfish across western Andalusia were sampled in late spring whereas those in DNS were sampled in early spring, the latter favouring Bd positives. In fact, all crayfish sampled within DNS in late spring (when water temperature can exceeded 25 ºC) were Bd negatives. But also, the great diversity and abundance of amphibian species in DNS (Díaz-Paniagua et al. 2006) could facilitate that this disease remains in the environment over time because amphibians vary greatly in their susceptibility to Bd (Scheele et al. 2017). We found relatively high prevalence of Bd in some ponds of DNS but no signs of chytridiomycosis in amphibian specimens and, as far as we know, no massive mortalities of amphibians have been detected in the area (Hidalgo et al. 2012). Lower infection intensity of Bd found in amphibians [reaching a maximum load of 68.0 ZE in one specimen of *P. walt*, which is far from the threshold of ~10,000 ZE to observe mass mortalities (Vredenburg et al. 2010)], could explain why the chytrid fungus is present in the area since long without relevant mass mortalities (Hidalgo et al. 2012). Another possible factor is related to the virulence of Bd, which depends on each strain (Rosenblum et al. 2013); however, nothing is known on which chytrid fungus strain is present in DNS.

Although some factors affecting Bd dynamics have been well studied (see paragraph above), how the fungus can be maintained in the environment remains less known. Some amphibian species, or some individuals within a species, may function as reservoirs of Bd since they are not highly susceptible to infection in spite of harbouring the pathogen and transmitting it to others (van Rooij et al. 2015; Scheele et al. 2017; Brannelly et al. 2018). But other studies in addition to ours have demonstrated infections of Bd in non-amphibian taxa such as reptiles (Kilburn et al. 2011), waterfowl (Garmyn et al. 2012; Johnson and Speare 2005), fish (Liew et al. 2017) and crayfish (McMahon et al. 2013; Brannelly et al. 2015a). The generalist strategy of the Bd fungus, which is able to infect a wide range of hosts, may have profound evolutionary consequences for the pathogen, including the evolution of highly pathogenic strains.

We show that the presence of the highly invasive red swamp crayfish could be a relevant factor in the prevalence of Bd in amphibians. However, our models for the infection intensity of Bd in amphibians showed that crayfish presence had no effect. Thus, other variables such as amphibian densities or environmental determinants (probably the hydroperiod) affecting in turn the viability of Bd zoospores, could be playing a more important role in Bd infection intensity. Our study demonstrates the presence of Bd in several amphibian species across DNS and a high prevalence of Bd in nearby sampling sites that had been studied ten years ago (Hidalgo-Vila et al. 2012). Therefore, the presence of the red swamp crayfish could explain the high prevalence of Bd, whereas pond desiccation in summer could prevent sudden declines in amphibian populations in DNS. Thus, human-made permanent ponds that supply water for livestocks within this protected area could act as infection hubs, not only because infected amphibians can remain there in summer (Hidalgo et al. 2012), but also because crayfish can co-occur there (Román 2014). If the red swamp crayfish acts as vector competent of Bd, it would be a good step in understanding the dynamic system of this disease in places where both taxa co-occur, and its participation in transmitting infection to amphibians (see references in van Rooij et al. 2015).

The positive relationship between the presence of the red swamp crayfish and prevalence of Bd in amphibians suggests an unrecognised indirect impact of crayfish on amphibian populations of DNS besides the already known direct effects (Arribas et al. 2014). Although the red swamp crayfish has been mainly translocated by human-mediated dispersal (Oficialdegui et al. 2019), its spread in this highly protected area seems to be due to the presence of seasonal streams connecting the ponds and human-made permanent ponds, thus facilitating the crayfish expansion (Román 2014). However, their overland movement in rice fields often depends on water availability, relative humidity, air temperature and time of the day (Ramalho & Anastácio 2015), so we can expect such behaviour in our study area, where temporary ponds are close to each other. Therefore, the particular system of DNS, which encompasses > 3,000 temporary waterbodies, could play a crucial role in the spread of the red swamp crayfish and, consequently, in the dynamics of Bd. More effort should be paid in controlling emerging diseases such as Bd and the factors influencing their dynamics in protected areas that are considered biodiversity hotspots.

To conclude, our study suggests a role of the red swamp crayfish as potential reservoir of the Bd pathogen and suitable predictor of its prevalence in amphibians. The suitability of the red swamp crayfish as a potential Bd reservoir (McMahon et al. 2013), together with its ubiquitous presence in temperate habitats worldwide (see Oficialdegui et al. 2019) and its positive relationship with high prevalence of Bd in amphibians, may represent new pathways of introduction for this disease globally. This study highlights the need to include non-amphibian hosts, especially invasive species such as the red swamp crayfish, as part of the dynamics of Bd infection in order to predict possible outbreaks of chytridiomycosis, as well as its contemplation in future conservation strategies on amphibians. Further research should consider experiments on the role of the red swamp crayfish in the transmission of Bd to amphibians, longitudinal studies to assess the effect of hydroperiod together with the presence of the red swamp crayfish simultaneously, as well as the inclusion of non-amphibian hosts in epidemiological models to estimate clinical outcomes of this panzootic disease.

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Tables

Table 1. The red swamp crayfish sampling in streams and ponds of western Andalusia, Spain, in 2015. For each location we show the coordinates, altitude (m), number of crayfish captured (n), type of habitat, prevalence of Bd (%) with 95% confidence intervals in parentheses, and infection intensity (Bd load in Zoospore Equivalents, mean ± SE).

Site	Code	Name	Lat	Long	Altitude	Habitat	n	Prevalence (CI)	Bd load
1	GDP	Guadalporcún	36.948	-5.3578	282	Stream	20	0	-
2	GUA	Guadiamar	37.657	-6.2290	203	Stream	20	0	-
3	HUE	Hueznar	37.933	-5.6971	422	Stream	20	0	-
4	MAN	Manecorro	37.124	-6.489	6	Lagoon	25	0	-
5	MAR	Martinazo	37.028	-6.438	8	Pond	20	0	-
6	STG	Guaperal	37.098	-6.4628	7	Pond	20	0	-
7	HOR	Hornueco	37.585	-6.5484	134	Stream	20	0	-
8	JAR	Jarrama	37.707	-6.4798	288	Stream	20	0	-
9	OLI	Olivargas	37.786	-6.8155	236	Stream	20	20.0 (5.7 – 43.7)	4.1 ± 1.2
10	VIL	Villar	37.688	-6.7254	229	Stream	19	0	-
11	VLV	Valverde	37.568	-6.7384	233	Stream	16	0	-
12	PAL	Palmones	36.286	-5.5949	122	Stream	20	0	-
13	VAL	Valle	36.084	-5.6933	58	Stream	20	0	-
14	YES	Yeso	36.371	-5.8415	22	Stream	7	0	-
							267	1.5 (0.4 – 3.8)	4.1 ± 1.2

Table 2. The red swamp crayfish and amphibian sampling in six ponds of Doñana Natural Space (south-western Spain) in 2018. For each location we show the coordinates, altitude (m), number of crayfish and amphibians captured (n), prevalence of Bd (%) with 95% Confidence Intervals in parentheses and infection intensity (Bd load in Zoospore Equivalents, mean ± SE).

							Crayfish			**Amphibians**		
Site	**Code**	**Location**	**Lat**	**Lon**	**Altitude**	***P. clarkii* presence**	**n**	**Prevalence (CI)**	**Bd load**	**n**	**Prevalence (CI)**	**Bd load**
1	ANS	Ánsares	37.122	-6.6046	23	yes	9	0.0 (0 – 33.6)	-	5	20.0 (0.5 – 71.6)	0.1 ± 0.0
2	OLL	Santa Olalla	36.981	-6.4731	9	yes	19	26.3 (9.1 – 51.2)	7.7 ± 2.3	40	57.5 (40.9 – 73.0)	9.3 ± 2.3
3	PAJ	Las Pajas	36.980	-6.4699	9	yes	9	22.2 (2.8 – 60.0)	1.7 ± 0.0	49	32.7 (19.9 – 47.5)	5.2 ± 4.2
4	TAR	Taraje	36.989	-6.4928	10	no	-	-	-	30	10.0 (2.1 – 26.5)	0.1 ± 0.0
5	ZAH	Zahillo	36.987	-6.5067	11	no	-	-	-	20	10.0 (1.2 – 31.7)	0.2 ± 0.1
6	PDR	P. del Raposo	36.996	-6.4932	8	no	-	-	-	21	4.8 (0.1 – 23.8)	0.1 ± 0.0
							37	18.9 (8.0 – 35.2)	6.0 ± 2.0	165	27.9 (21.2 – 35.4)	1.9 ± 0.6

Table 3. The seven amphibian species sampled in Doñana Natural Space in 2018. Life stage is represented by juveniles (J) and adults (A). The grey area includes urodelan species (salamanders) whereas the white area includes anuran species (frogs). Prevalence of Bd (%) with 95% Confidence Intervals in parentheses and Bd loads on amphibians (Zoospore Equivalents, mean ± SE) are shown.

Species	**n**	**Life Stage (J/A)**	**Prevalence (CI)**	**Bd load**
Pleurodeles waltl	46	29/17	39.1 (25.1 – 54.6)	5.7 ± 3.8
Triturus pygmaeus	77	0/77	35.1 (24.5 – 46.8)	7.2 ± 2.1
Discoglossus galganoi	2	0/2	0.0 (0 – 84.2)	-
Epidalea calamita	4	1/3	0.0 (0 – 60.2)	-
Hyla meridionalis	29	0/29	0.0 (0.0 – 11.9)	-
Pelobates cultripes	5	3/2	0.0 (0 – 52.2)	-
Pelophylax perezi	2	2/0	50.0 (1.3 – 98.7)	0.1

Figure Captions

Fig. 1 (A) Map of the study area showing crayfish sampling points in streams of three provinces in western Andalusia (“HUE” Huelva, “SEV” Seville and “CAD” Cadiz). Black spots indicate locations with Bd- and the black triangle shows the only Bd+ location (see sites in Table 1). Area depicted by a square indicates the localized highly protected area of Doñana Natural Space (DNS) and greys represent the Doñana Natural Park (light grey) and Doñana National Park (dark grey). (B) Protected areas of Doñana Natural Space (Doñana Natural Park and Doñana National Park). Sampling sites with crayfish (black squares) and ponds without crayfish (black triangles) are numerated as follows: (1) ANS, (2) OLL, (3) PAJ, (4) TAR, (5) ZAH, and (6) PDR (see Table 2 for details). Dark grey patches depict temporary ponds and grey circles depict permanent water bodies.

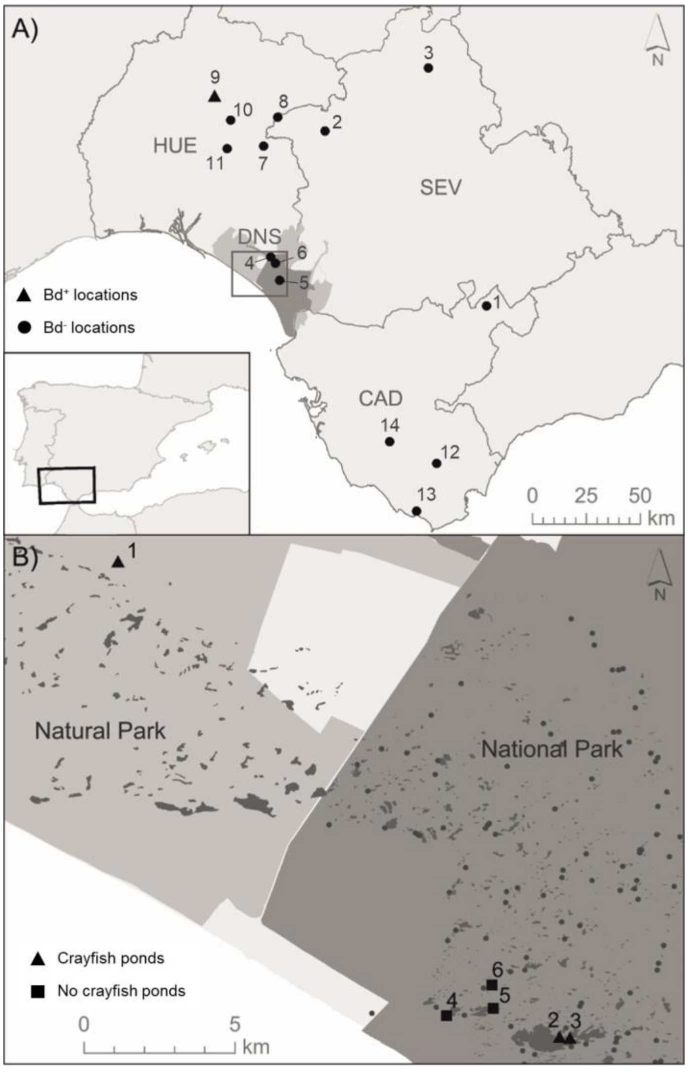

Fig. 2 Section of GI tissue from one adult Bd+ red swamp crayfish, Procambarus clarkii, at different scales. Black arrows point out two different zoosporangia containing zoospores of Bd. A) The two zoosporangia at 50 µm; B) the two zoosporangia at 20 µm; C) one of the two zoosporangia at 10 µm; and D) the other zoosporangium at 10 µm.

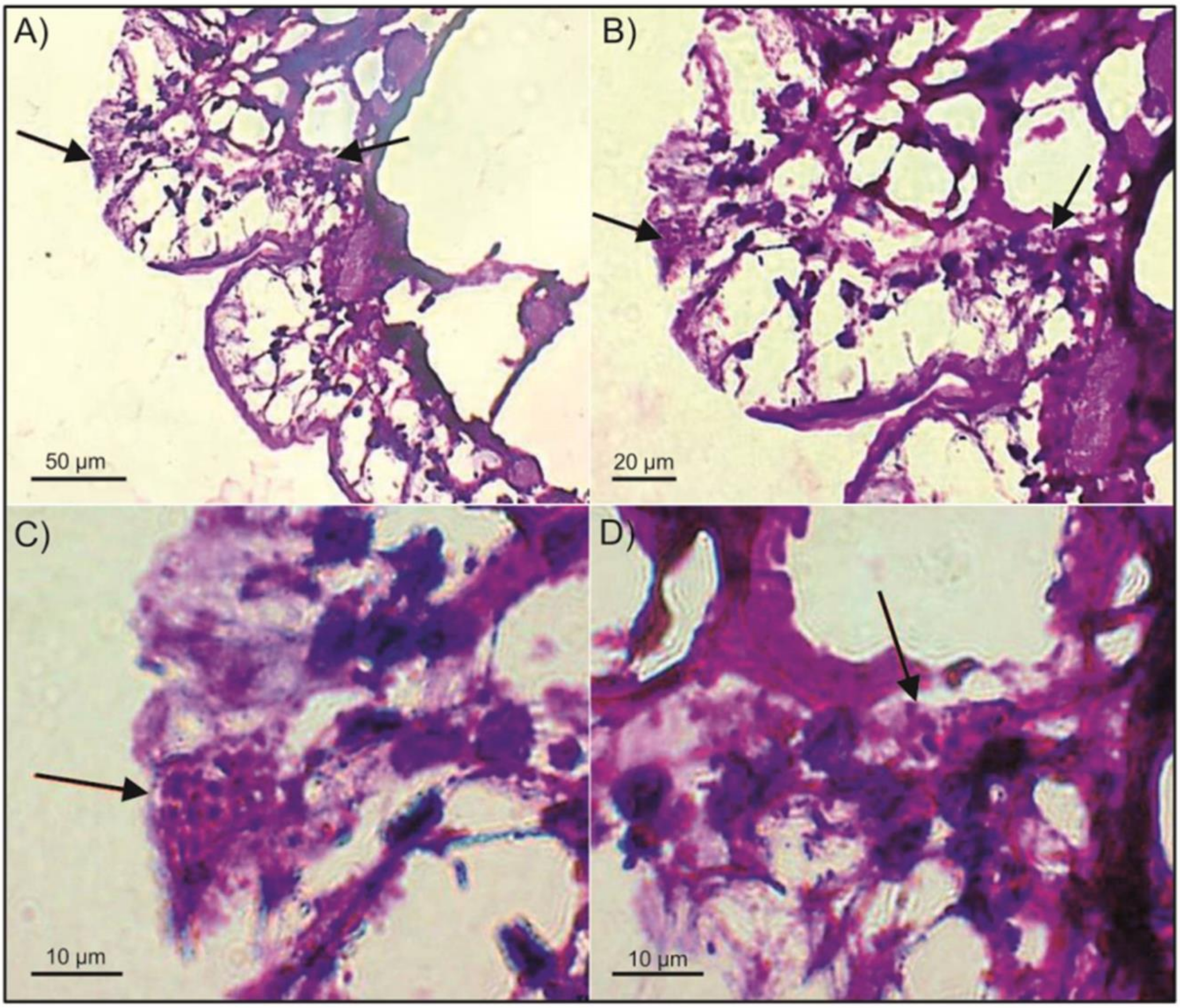

Fig. 3 (A) Prevalence and (B) infection intensity of Bd in amphibian species from six sampled ponds in Doñana Natural Space. The grey area includes ponds with presence of the red swamp crayfish. In figure 3A, while the number of Bd-negative are depicted in white bars, Bd-positive amphibians are depicted in black bars.